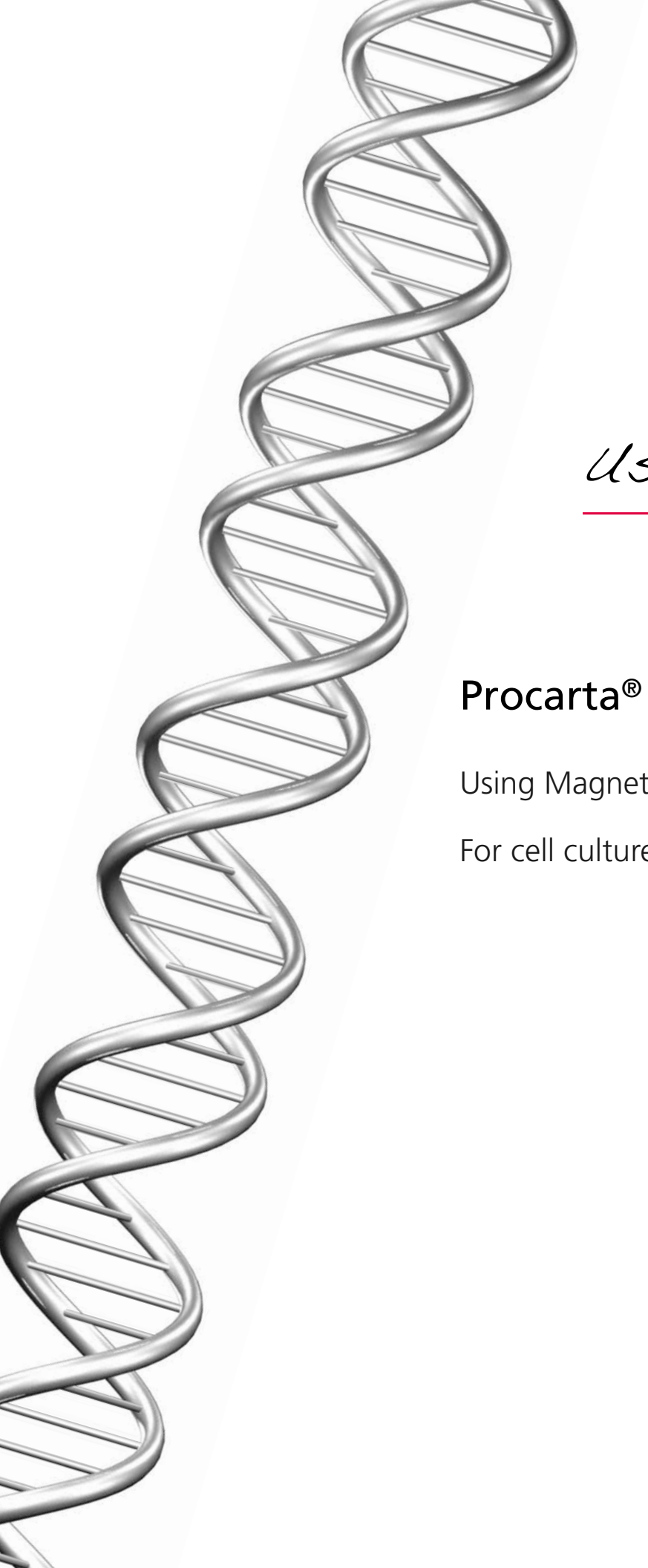


Affymetrix® | Panomics Solutions

User Manual

Procarta® Immunoassays

Using Magnetic Beads

For cell culture supernatant samples.

P/N 17992-CC Rev.A 020912

For research use only.
Not for use in diagnostic procedures.

Trademarks

Affymetrix® , and Procarta® are registered trademarks of Affymetrix, Inc.

Luminex®, xMAP®, and xPonent®are registered trademarks of the Luminex Corporation.

All other trademarks are the property of their respective owners.

Limited License

Subject to the Affymetrix terms and conditions that govern your use of Affymetrix products, Affymetrix grants you a non-exclusive, non-transferable, non-sublicensable license to use this Affymetrix product only in accordance with the manual and written instructions provided by Affymetrix. You understand and agree that, except as expressly set forth in the Affymetrix terms and conditions, no right or license to any patent or other intellectual property owned or licensable by Affymetrix is conveyed or implied by this Affymetrix product. In particular, no right or license is conveyed or implied to use this Affymetrix product in combination with a product not provided, licensed, or specifically recommended by Affymetrix for such use.

Citing Procarta® Immunoassay in Publications

When describing a procedure for publication using this product, please refer to it as the Procarta® Immunoassay from Affymetrix.

Disclaimer

Affymetrix, Inc. reserves the right to change its products and services at any time to incorporate technological developments. This manual is subject to change without notice.

Although this manual has been prepared with every precaution to ensure accuracy, Affymetrix, Inc. assumes no liability for any errors or omissions, nor for any damages resulting from the application or use of this information.

Copyright

© 2012 Affymetrix Inc. All rights reserved.

Contents

Intended Use 1
Contacting Technical Support 1
About Procarta Immunoassay Kits 1
How it Works 2
Procarta Immunoassay Kit Contents and Storage Conditions 3
Required Equipment and Materials Not Supplied 4
Precautions and Technical Hints 4
Sample Preparation 5
Preparing Antigen Standards 6
Performing the Assay 7
Setup of the Luminex Instrument 9
Analyzing Results 9
Troubleshooting 10
Validating the Hand-Held Magnetic Plate Washer 12
Example Plate Layout 13
Blank Plate Layout 13

Intended Use

This user manual is for a Procarta® Immunoassay Kit - Magnetic Beads from Affymetrix to perform quantitative, multiplexed immunoassays based on the Luminex® technology. The procedure is for simultaneous measurements of multiple protein biomarkers in cell culture supernatant samples. The assay protocol and reagents supplied are not compatible with other manufacturer's reagents. Each 96-well plate kit is configured to allow for the following usage: 16 wells for an 8-point standard curve (in duplicate), 2 wells for blanks, and up to 78 wells for samples. Procarta Immunoassay kits can be stored for up to 6 months from the date of receipt when stored at recommended temperatures.

NOTE: For the most current version of user documentation, go to our website at www.panomics.com

Contacting Technical Support

For technical support, contact the appropriate resource provided below based on your geographical location. For an updated list of FAQs and product support literature, visit our website at www.panomics.com.

Location	Contact Information
North America	1-877-726-6642 option 1, then option 3; pqbhelp@affymetrix.com
Europe	+44 1628-552550 techsupport_europe@affymetrix.com
Asia	+81 3 6430 430 techsupport_asia@affymetrix.com

About Procarta Immunoassay Kits

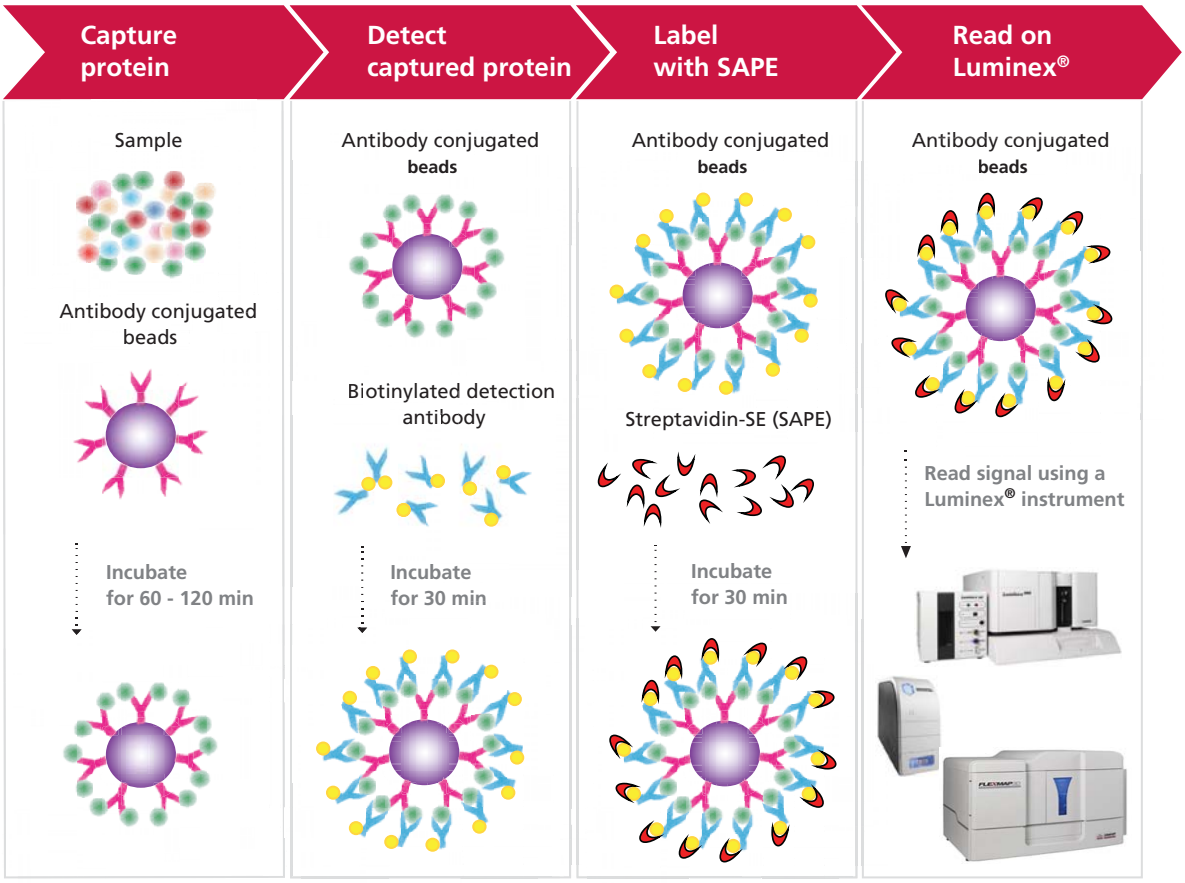
Procarta Immunoassay Kits are available as:

- Standard pre-mixed panels
- “By Request” user configured panels
- New custom assay development for analytes not listed on our website

Procarta Immunoassay Kits contain all the reagents required to run the assays. Please contact your local Affymetrix sales representative for new custom assay development for analytes not listed on our website www.panomics.com.

How it Works

Procarta® Immunoassays use the xMAP® technology (multi-analyte profiling beads) to enable the detection and quantitation of multiple protein targets simultaneously in diverse matrices. The xMAP system combines a flow cytometer, fluorescent-dyed microspheres (beads), dual laser design and digital signal processing to effectively allow multiplexing of up to 100 unique assays within a single sample. The Procarta Immunoassay kits are compatible with all Luminex and Luminex-based instruments currently available.



Procarta Immunoassay Kit Contents and Storage Conditions

The Procarta Immunoassay Kit contains the following components listed below. The kits are available in single 96-well plate or ten 96-well plate formats. Refer to the Package Insert for quantities and details of components supplied. The kits are shipped with blue ice. Shelf-life of the kit is 6 months from date of receipt when stored at 2-8 °C. The kits are also supplied with the following inserts:

- **Packaging Insert**: Describes the products included in the kit
- **Premixed Antigen Standard Insert**: Lists the lot number and reconstituted standard values.
- **Bead Analyte Association Insert**: Lists the bead region with the corresponding analyte.

Component	Description
Antigen Standards, premixed, lyophilized (2 vials each lot for a 1 plate kit)	Recombinant proteins in lyophilized powder. Do not reuse, discard after use. Please note that more than 1 lot of vials may be shipped with each kit. Review the Premixed Standard Insert prior to use.
Detection Antibody, premixed	Detection antibodies in aqueous buffered solution
Antibody Magnetic Beads	Capture antibodies conjugated to microspheres in aqueous buffered solution. The Bead ID's are printed on the inside flap of the Kit Box.
Streptavidin-PE (SAPE)[1]	Streptavidin-conjugated R-phycoerythrin in aqueous buffered solution
10X Wash Buffer[1]	Concentrated aqueous buffered solution
Reading Buffer[1]	Aqueous buffered solution
PCR 8-Tube Strip	0.2 mL polypropylene PCR 8-tube strip
Procarta 96-well Flat Bottom Plate	96-well, flat bottom microplate
Plate Seals	Adhesive-backed foil plate sealer

[1] Contains *sodium azide. See WARNING below.*

WARNING: All chemicals should be considered potentially hazardous. We recommend that this product and its components be handled by those trained in laboratory techniques and be used according to the principles of good laboratory practice.

WARNING: This kit contains small quantities of sodium azide. Sodium azide is highly toxic and reactive in the pure form. At this product's concentration, though not classified as hazardous, build up of sodium azide may react with lead and copper plumbing to form highly reactive explosive metal azide. Dispose of the product in accordance with all State and local regulations.

Required Equipment and Materials Not Supplied

Required Equipment/Material	Source	Part Number
Hand-Held Magnetic Plate Washer	Affymetrix	QP0702
Microplate shaker	Labline	4625 or equivalent (must have 3 mm orbit with ability to maintain 500 rpm)
Luminex or Luminex-based instrument	MiraiBio, Bio-Rad or other Luminex instrument providers	
Vortex mixer	Major laboratory supplier (MLS)	
Adjustable single and multi channel precision pipettes for dispensing 1-20 µL, 20-200 µL and 200-1000 µL	MLS	
Reagent reservoirs, 25 mL and 100 mL capacity	Vist Labs Corning Costar	3054-1002 or equivalent CLS 4873 or equivalent
Double-distilled (dd) water (H_2O)	MLS	
Microcentrifuge	MLS	

Precautions and Technical Hints

- Thoroughly read this user manual and product insert that is included with the assay kit. The product insert may contain specific instructions for proper use of the “By Request” or custom panels.
- Before starting the assay, turn on the Luminex machine and initiate the startup protocol. It takes 30 min for the lasers to warm-up. Make sure the Luminex machine is calibrated according to the manufacturer’s instructions.
- When working with samples and standards, change the pipette tips after every transfer and avoid creating bubbles when pipetting.
- During the incubation steps, cover the 96-well plate with aluminum foil to minimize exposure of the beads to light.
- Be careful not to invert the Procarta 96-well Flat Bottom Plate during the assay or allow contents from one well to mix with another well.
- Ensure that the Hand-Held Magnetic Plate Washer is securely locked into place prior to inverting when performing the wash steps.
- Use a multi-channel pipette and reagent reservoirs whenever possible to achieve optimal assay precision.
- Store the detection antibody, antibody beads, Streptavidin-PE, samples, and reconstituted standards (including standard diluents sets) on ice before adding to the 96-well plate.
- For frozen samples, thaw completely on ice and mix well prior to running the assay.

Sample Preparation

Cell culture supernatant samples can be obtained from adherent and non-adherent cells. A total volume of 50 µL/well of cell culture supernatant is needed and a minimum of 2 replicates is recommended.

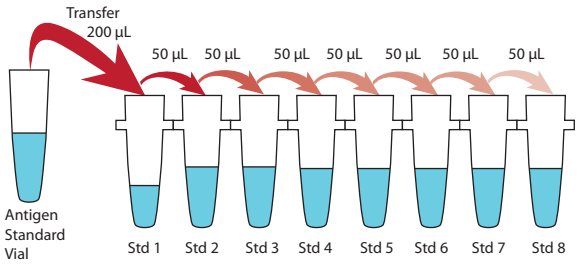
Some samples may contain high concentrations of the analytes. Dilution of the samples may be needed if the analyte concentration is above the assay upper limit of quantitation. Serial dilution of the samples may need to be prepared to determine the appropriate dilution factor for accurately measuring the analytes of interest. Use the same fresh culture medium used for culturing the experimental cells to prepare dilutions of the samples.

If the sample contains cells, centrifuge the sample at 300 x g for 5 min at 2-8 °C. Collect the supernatant. Use immediately or aliquot and store at -80 °C.

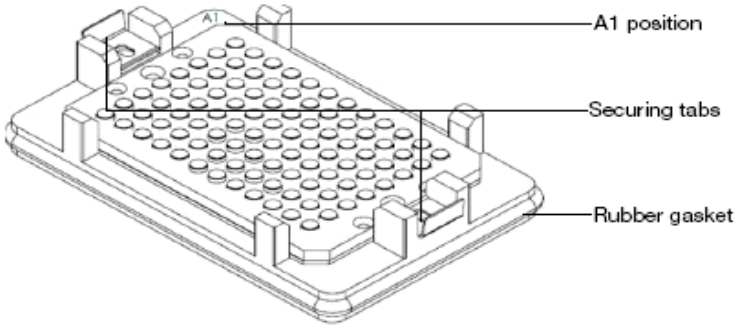
The TGF-β assay requires a special sample preparation procedure. The TGF- β assay can only detect the active form of TGF- β. The samples must be acid treated and then neutralized to convert the complexed form of TGF- β to its active form. The assay should be processed as a single plex assay since the sample must be acid treated. The TGF-β sample preparation protocol can be found on our website: www.panomics.com.

Preparing Antigen Standards

This section provides instructions on how to make a 4-fold, 8-point standard curve for the assay panel. The antigen standards should be prepared after sample preparation is completed. The serially diluted antigen standards should be added to the assay plate at the same time the samples are added. Each1 plate kit is shipped with two vials of identical antigen standards from the same lot. In some cases an additional set(s) of standards from a different lot may be included in the kit. Please refer to the **Premixed Antigen Standard Insert** when assigning the Standard 1- Standard 8 antigen values for each analyte.

Step	Action
Step 1. Reconstitute Lyophilized Antigen Standards	**A.** Instructions for assay panels with only 1 standard lot in the kit: 1. Centrifuge the antigen standard vial at 2000 x *g* for 10 sec. 2. Add 250 µL of fresh cell culture media into the vial. 3. Vortex gently for 30 sec. 4. Incubate on ice for 5-10 min. **B.** Instructions for assay panels with more than one standard lots in the kit: 1. Centrifuge all the antigen standard vials with different lot numbers at 2000 x *g* for 10 sec. 2. Add 250 µL of fresh cell culture media into one of the vials. 3. Incubate the vial on ice for 5-10 min. 4. Vortex gently for 30 sec. 5. Transfer the entire content into the second vial with a different lot number. 6. Incubate on ice for 5-10 min. 7. Vortex gently for 30 sec. 8. If more than 2 lots of antigen standards are in the kit, repeat steps 5-7 until all the vials with different lot numbers are reconstituted. **NOTE:** **For panels with liquid antigens, follow the instructions on the Package Insert.**
Step 2. Prepare 4-Fold Serial Dilution	**A.** Prepare a 4-fold serial dilution of the reconstituted standard(s) using the PCR 8-tube strip provided. **B.** Add 200 µL of the reconstituted antigen standards into the first tube of the strip tube and label as Standard 1 (Std 1). **C.** Add 150 µL fresh cell culture media into Tubes 2-8. **D.** Using a P-200 pipette, transfer 50 µL of the reconstituted antigen standards from Tube 1 into Tube 2. **E.** Mix by pipetting up and down for a total of 10 times. **F.** After changing the pipette tip, transfer 50 µL of the mixed standards from Tube 2 into Tube 3 **G.** Mix by pipette up and down 10 times. **H.** Repeat Actions D to G for the rest of the tubes to prepare Std 4-8. 

Performing the Assay

Step	Action
Step 1. Prepare 1X Wash Buffer	Bring the 10X Wash Buffer to room temperature and vortex for 15 seconds. Mix 20 mL of the 10X Wash Buffer with 180 mL ddH20. **NOTE: 1X wash Buffer can be stored at 2-8 °C for up to 6 months. Bring the buffer to room temperature prior to use.**
Step 2. Prepare the Procarta 96-Well Flat Bottom Plate	Mark the standard, sample and blank wells. For your convenience, a blank layout is provided in the Plate Layout section.
Step 3. Add the Antibody Magnetic Beads	**A.** Vortex the Antibody Magnetic Beads for 30 sec. **B.** Add 50 µL of the Antibody Magnetic Beads to each well. **C.** Insert the Procarta 96-Well Flat Bottom Plate into the Hand-Held Magnetic Plate Washer so that the A1 location of the 96-Well Plate matches up with the A1 on the washer. **D.** Lock the 96-Well Plate in place by pushing the 2 securing tabs, located on each end of the washer, towards the 96-Well Plate until they overlap the skirt of the 96-Well Plate. **E.** Verify that the 96-Well Plate is securely locked by holding the assembly in the palm of your hand and gently pulling up on the 96-Well Plate. **F.** Wait 2 min to allow the Antibody Magnetic Beads to accumulate on the bottom of each well. **G.** Remove the liquid in the wells by quickly inverting the Hand-Held Magnetic Plate Washer and 96-Well Plate assembly over a sink or waste container. Do not remove the 96-Well Plate from the Hand-Held Magnetic Plate Washer. Blot the inverted assembly onto several layers of paper towels to remove any residual solution. 
Step 4. Wash Antibody Magnetic Beads	**A.** Add 150 µL of 1X Wash Buffer into each well. **B.** Wait 30 sec to allow the Antibody Magnetic Beads to accumulate on the bottom of each well. **C.** Remove the wash buffer in the wells by quickly inverting the Hand-Held Magnetic Washer and 96-Well Plate assembly over a sink or waste container. **D.** Blot the inverted assembly onto several layers of paper towels to remove any residual solution.
Step 5. Add Antigen Standards and Samples	Add 50 µL of standards or 50 µL of samples as marked on the plate layout sheet into each well. For blanks, add 50 µL of cell culture media.

Step	Action
Step 6. Incubate the 96-Well Plate	**A.** Seal the 96-Well Plate using a Plate Seal provided. **B.** Remove the 96-Well Plate from the Hand- Held Magnetic Plate Washer and completely wrap the 96-Well Plate with aluminum foil. **C.** Room temperature incubation: 1. Shake the 96-Well Plate at 500 rpm for 60 to 120 min at room temperature. 2. Proceed to step 7. **D.** Alternatively, the 96-Well Plate can be incubated overnight. 1. Shake the 96-Well Plate at room temperature for 30 min. 2. Transfer the plate to 4 °C and store on a level surface. 3. After incubation, remove the 96-Well Plate from 4 °C and shake for 30 min at room temperature. 4. Proceed to step 7. **NOTE:** We recommend 120min at room temperature or overnight incubation at 4 °C for assays that require higher sensitivities.
Step 7. Wash the 96-Well Plate	**A.** Insert the 96-Well Plate into the Hand-Held Magnetic Plate Washer and wait 2 min to allow the Antibody Magnetic Beads to accumulate on the bottom of each well. **B.** Carefully remove the Plate Seal to avoid splashing the plate contents. **C.** Remove the supernatant in the wells by quickly inverting the Hand-Held Magnetic Plate Washer and 96-Well Plate assembly over a sink or waste container. **D.** Add 150 µL of 1X Wash Buffer into each well. **E.** Wait 30 sec to allow the Antibody Magnetic Beads to accumulate on the bottom of each well. **F.** Remove the supernatant in the wells by quickly inverting the 96-Well Plate over a sink or waste container. Repeat Actions D-F 2 more times for a total of 3 washes. **G.** After the last wash, blot the assembly onto several layers of paper towels to remove any residual solution. **NOTE: When washing the 96-Well Plate, we recommend using a multi-channel pipette or a multi-channel automatic liquid dispenser. Avoid touching the pipette tips to the sides of the wells when adding wash buffer using a multi-channel pipette.**
Step 8. Add Premixed Detection Antibodies	**A.** Add 25 µL of Detection Antibodies into each well. **B.** Seal the 96-Well Plate with a new Plate Seal. **C.** Remove the 96-Well Plate from the Hand-Held Magnetic Plate Washer. **D.** Wrap the 96-Well Plate with aluminum foil and shake at 500 rpm for 30 min at room temperature.
Step 9. Wash the 96-Well Plate	**A.** Repeat Step 7.
Step 10. Add SAPE	**A.** Vortex the SAPE solution vial for 20 sec. **B.** Add 50 µL of SAPE solution into each well. **C.** Seal the 96-Well Plate with a new Plate Seal. **D.** Remove the 96-Well Plate from the Hand-Held Magnetic Plate Washer. **E.** Wrap the 96-Well Plate with aluminum foil and shake at 500 rpm for 30 min at room temperature.
Step 11. Wash the 96-Well Plate	**A.** Repeat Step 7.

Step	Action
Step 12. Prepare the 96-Well Plate for Analysis on a Luminex Instrument	**A.** Add 120 µL of the Reading Buffer into each well. **B.** Seal the 96-Well Plate with a new Plate Seal. **C.** Remove the 96-Well Plate from the Hand-Held Magnetic Plate Washer. **D.** Wrap the 96-Well Plate with aluminum foil and shake at 500 rpm for 5 min at room temperature. **E.** Remove the Plate Seal prior to reading on the Luminex instrument. **NOTE:** **We recommend reading the assayed samples on the Luminex instrument immediately. However, the 96-Well Plate can be wrapped with aluminum foil and stored for up to 48 hours at 4 °C before proceeding. After storage, shake at 500 rpm for 5 min at room temperature prior to reading. Delay in reading the assayed samples may result in decreased sensitivities for some assays.**

Setup of the Luminex Instrument

Sample Size	DD Gate	Timeout	Bead Event/Bead Region
100 µL	5,000 - 25,000	45 sec	50-100

If you are running assays on your Luminex instrument that uses both Plates for Magnetic Beads and Filter Plates for Polystyrene Beads, verify the probe height for each plate type before reading. Failure to adjust the probe height can cause damage to the instrument.

The Luminex system allows for calibration of Low and High RP1 target values. We recommend RP1 Low target value settings for Procarta Immunoassays.

Please refer to the label inside of the Kit Box or the **Bead Analyte Association Insert** for bead region-analyte associations when entering the information into the Luminex acquisition software (xPonent®, Bio-Plex®, MasterPlex®, StarStation®).

Please also refer to the **Premixed-Antigen Standard Insert** when assigning the Standard 1 (Std 1) concentration into the analysis software. Each analyte may have a different Std 1 concentration. For example if the starting concentration was 20,000, then a 4 fold dilution for Std 1-8 would be 20,000, 5000. 1250, 312, 78, 19.5, 4.8 and 1.2 pg/ml.

NOTE: **If there is a malfunction of the Luminex instrument or software during the run, the 96-Well Plate can be re-read. Remove the 96-Well Plate from the instrument, insert the 96-Well Plate into the Hand-Held Magnetic Plate Washer, wait 2 min, then remove the buffer in the wells by quickly inverting the 96-Well Plate over a sink or waste container. Blot the assembly onto several layers of paper towels to remove any residual solution. Resuspend the beads in 120 µL of Reading Buffer, remove from the Hand-Held Magnetic Plate Washer, seal the 96-Well Plate with a new Plate Seal and shake at 500 rpm for 5 min at room temperature. The assayed samples may take longer to read since there will be less beads in the wells.**

Analyzing Results

The concentration of the samples can be calculated by plotting the expected concentration of the standards against the MFI generated by each standard. A 4PL or 5PL algorithm is recommended for the best curve fit.

Analyze the assayed samples according to the operation manual for the Luminex or Luminex-based instrument.

Troubleshooting

Observation	Probable Cause	Recommend Solution
Low Flow Rate	Partial Blockage of the flow cell	Remove the 96-well plate and perform a wash and rinse cycle.
	Instrument needle is partially clogged	Replace or clean needle according to the manufacturer's recommendations.
High CVs	Samples and antigen standards not stored on ice	Prepare the samples and standards on ice before setting up the assay.
	Contamination from re-using the Plate Seal	Use a new Plate Seal for each incubation step.
	Incomplete washing	Blot the 96-Well Plate onto several layers of paper towels to remove any residual solution after each wash step.
	Contamination from contents from adjacent wells	Avoid splashing the Wash Buffer during wash steps into adjacent wells
	Poor pipetting techniques	Use appropriate pipetting techniques. Use new pipette tips for each well during sample and standard addition. Avoid touching pipette tips to sides of the wells when adding wash buffer.

Observation	Probable Cause	Recommend Solution
Low bead count	Probe height is incorrect	Refer to the Luminex manual for proper adjustment of the needle height.
	Reading buffer volume added in the last step to resuspend the beads is too low	Add 120 µL Reading Buffer into each well and shake at 500 rpm for 5 min at room temperature to resuspend the beads prior to reading on the Luminex instrument. Make sure sample size is set at 100 µL in the acquisition protocol.
	High bead aggregation	Vortex the bead suspension well before using in the assay and ensure that the beads are properly mixed during the incubation steps.
	Dyes contained in the beads are photo-bleached from overexposure to light	Store bead solution in the dark and protect the 96-Well Plate from light by wrapping the 96-Well Plate with aluminum foil.
	Partial blockage of the flow cell	Remove the 96-Well Plate and perform a wash and rinse to the instrument
	Instrument needle is partially clogged	Replace or clean needle according to the manufacturer's recommendations
	Beads settle on the bottom of the well	Confirm that the plate shaker is set to 500 rpm and shaking for at least 5 min before reading.
	Did not use supplied 96-well microtiter plate	Only use the Procarta 96-Well Flat Bottom Plate supplied with the kit
	Air bubble in the sample loop	Refer to the Luminex manual for proper removal of the air bubble.
Low signal or sensitivity	Standards not reconstituted and diluted correctly	Prepare fresh antigen standards following the instructions in the Preparing Antigen Standards Section.
	Expired reagents were used	Reagents are good for 6 months from the date of receipt. Do not use expired reagents.
	Suboptimal assay conditions	Follow the recommended incubation times and temperature. Shake the 96-Well Plate during all incubations except during optional overnight incubation step (Step 7D).
Poor accuracy	Did not use the appropriate assay diluents	Use the same sample type-specific standard and assay buffers for standard and sample preparations.
	Samples and antigen standards were not stored on ice	Prepare and store the samples and standards on ice before setting up the assays

Validating the Hand-Held Magnetic Plate Washer

The Hand-Held Magnetic Plate Washer is designed for use with the Procarta Immunoassay configured for the Procarta 96-Well Flat Bottom Plate. This device uses magnetic separation to enable quick and easy processing of wash steps after each incubation. This section describes how to validate the hand held washer prior to running an experiment and how to operate the device when performing washes.

IMPORTANT: **Do not substitute another plate type for the Procarta 96-Well Flat Bottom Plate (Catalog No: PC0169) included in the Procarta Immunoassay Kit. This plate is specifically for use with the Hand-Held Magnetic Plate Washer (Catalog No: QP0702). Other plate types can result in assay failure or poor assay performance. After reading all instructions in this document, we recommend that you perform a series of practice washes using the Hand-Held Magnetic Plate Washer**

Step	Action
Step 1. Set Up Luminex Instrument	Set up the Luminex instrument according to the guidelines provided. Define a protocol with the appropriate bead regions and set to read 2 wells. Refer to the Procarta Immunoassay Kit Product Insert for the target-bead population of the panel.
Step 2. Prepare 96-Well Plate	Vortex Antibody Magnetic Beads. Add 50 µL of the Antibody Magnetic Beads into each of 2 wells on the 96-Well Flat Bottom Plate.
Step 3. Perform Wash Steps	**A.** Perform a series of wash steps using the Wash Buffer to simulate the multiple wash steps in the assay. No incubation needed. **B.** After the final wash step, add 120 µL Reading Buffer to each well. Cover the 96-Well Flat Bottom Plate with a plate seal, place on a shaking platform at room temperature and shake for 2 to 5 min to completely resuspend the Antibody Magnetic Beads.
Step 4. Read 96-Well Flat Bottom Plate	**A.** Insert the 96-Well Flat Bottom Plate into the instrument and read the 2 wells. **B.** View the window with the bead regions and Doublet Discriminator (DD) gate. The expected results are: ▪ Signals for the expected beads should show up on the bead map ▪ Average bead count should be greater than 50/region ▪ The main single peak in the DD gate window should be within the set DD gates **NOTE:** **If you are running assays on your Luminex instrument that uses both Plates for Magnetic Beads and Filter Plates for Polystyrene Beads, verify the probe height for each plate type before reading.**

Example Plate Layout

Standards		Samples									
Standard 1	Standard 1	1	1	8	8	16	16	24	24	32	32
Standard 2	Standard 2	2	2	9	9	17	17	25	25	33	33
Standard 3	Standard 3	3	3	10	10	18	18	26	26	34	34
Standard 4	Standard 4	4	4	11	11	19	19	27	27	35	35
Standard 5	Standard 5	5	5	12	12	20	20	28	28	36	36
Standard 6	Standard 6	6	6	13	13	21	21	29	29	37	37
Standard 7	Standard 7	7	7	14	14	22	22	30	30	38	38
Standard 8	Standard 8	Blank	Blank	15	15	23	23	31	31	39	39

Blank Plate Layout

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B												
C												
D												
E												
F												
G												
H												